(51) International Patent Classification 3 :		(11) International Publication Number: WO 82/04443
C12P 21/00, 21/02; C12N 15/00	A1	(43) International Publication Date: 23 December 1982 (23.12.82)

(54) Title: GENETIC TRANSFORMATION OF ZYGOTES

(57) Abstract

Genetic transformation of a zygote and the embryo and mature organism which result therefrom is obtained by placing or inserting exogenous genetic material into the nucleus of the zygote or into any genetic material which ultimately forms at least a part of the nucleus of the zygote. It is preferred that the exogenous genetic material be added to a pronuclei of the zygote and is particularly preferred that it be added to the male pronucleus of the zygote. Thereafter, the zygote is allowed to undergo differentiation and development into the organism. The genotype of the zygote and the organism which results therefrom will include the genotype of the exogenous genetic material and the exogenous genetic material will be phenotypically expressed. The invention can be utilized in a variety of ways including, for example, animal and plant breeding to modify or create new species, it can be used in epigenetics and in the understanding and treatment of genetic diseases.

-1-

Description

GENETIC TRANSFORMATION OF ZYGOTES

Technical Field:

The present invention relates to the field of genetics
and methods of altering the genetic material of an organism.
The invention has a wide variety of applications, for example,
in animal and plant breeding, including transgenosis, in the
field of epigenetics, which deals with the variation in gene
expression during differentiation and development of
eucaryotic organisms, and in the understanding and
treatment of genetic diseases.

Background Art:

Prior Art Statement

The possibility of transferring specific functional genes
from one species to another has intrigued scientists for
years. However, such transfers have only been realized in
cell culture systems. Cloned rabbit β-globin gene sequences
have been stably introduced into thymidine kinase (TK)
deficient mutant mouse cells by DNA mediated cotrans-
formation using the rabbit β-globin gene and herpes virus TK
gene sequences, Wigler, M., et al, Cell, 11:223-232 (1977).
This approach has been extended to use cellular TK, Wigler,
M., et al, Cell, 14:725-731 (1978), adenine phoshoribosyl
transferase, Graf, L.H., et al, Som. Cell Genet., 5:1031-1044
(1979), genes as unlinked but selectable markers.
Additionally, the use of a mutant hamster gene, which codes
for an altered dihydrofolate reductase, as a selectable marker
offering methotrexate resistance, allows the introduction and
amplification of a broad range of genetic elements into a
variety of cell lines, Wigler, M., et al, Proc. Natl. Acad. Sci.
U.S.A., 77:3567-3570 (1980). Although these
transformation experiments enable a direct gene transfer

between mammalian species to be effected, these techniques are all restricted to cell culture systems wherein only one cell in 10^5-10^7 is transformed which requires that the transformed cells be selected for on restrictive media.

5   The direct microinjection of DNA has been used to introduce Herpes Simplex virus TK gene into cultured mammalian cells. Capecchi, M., Cell, 22:479-488 (1980) However, the efficiency of transformation was extremely low even when the small pBR 322/TK DNA was coinjected with SV40 DNA, i.e., 15 transformants per 1,000 cells injected. Additionally, this technique does not result in the production of a mature organism much less one that could exhibit a phenotypic alteration.

       Mosaic mice have been constructed by the injection of teratocarcinoma cells into the blastocysts of developing mice. Brinster, R.L., J. Exp. Med., 140:1049-1056 (1974), Mintz, B., et al, Proc. Natl. Acad. Sci. U.S.A., 72:3585-3589 (1975) and Papaioannou, V.E., et al, Nature 258:69-73 (1975). Teratocarcinoma cells have also been used as vehicles for introducing genes into mice to produce mosaic mice. Pellicer, A., et al, Proc. Natl. Acad. Sci. U.S.A. Sci. U.S.A., 75:5113-5117 (1978) and Illmensee, K., et al Proc. Natl. Acad. Sci. U.S.A., 75:1914-1981 (1978). However, by definition, a mosaic mouse has patches of different, mutually exclusive genotypes and/or phenotypes. Additionally, the possibility of germ-line transmission with mosaic mice is reduced due to the fact that teratoma cells of XX chromosomal constitution cannot make sperm in mice that develop as males.

       Recently, Gordon, et al, Proc. Natl. Acad. Sci. U.S.A., 77:7380-7384 (1980), have observed TK genes in mice developed from embryos microinjected with the TK gene incorporated into a chimeric SV40 viral vehicle. These mice, however, did not express the TK gene product. In these

experiments, as well as the experiments relating to the use of teratocarcinoma cells, no phenotypic alteration of the test animal was accomplished. The results reported by Gordon, et al, are not surprising since the SV40 virus has previously been shown to be functionally and genetically inactive when introduced into mouse embryos. Jaenisch, R., et al, Proc. Natl. Acad. Sci. U.S.A., 71:1250-1254 (1974).

Disclosure of the Invention:

Genetic transformation of a zygote and the embryo and mature organism which result therefrom is obtained by the placing or insertion of exogenous genetic material. Additionally, the inclusion of the exogenous genetic material in the zygote will result in a phenotypic expression of the exogenous genetic material. The genotype of the exogenous genetic material is expressed upon the cellular division of the zygote. However, the phenotypic expression, e.g., the production of a protein product or products of the exogenous genetic material or alteration of the zygote's or organism's development during which the particular exogenous genetic material is active. Alteration of the expression of a phenotype includes an enhancement or dimishment in the expression of a phenotype or an alteration in the promotion and/or control of a phenotype including the addition of a new promotor and/or controller or supplementation of an existing promotor and/or controller of a phenotype.

The present invention has application in the genetic transformation of multicellular eucaryotic organisms which undergo syngamy, i.e., sexual reproduction by the union of gamete cells. Examples of such organisms include amphibians, reptiles, birds, mammals, bony fishes, cartilaginous fishes, cyclostomes, arthropods, insects, mollusks, thallophytes, embryophytes including gymnosperms and angiosperms. Preferred organisms include mammals,

The invention is particularly useful in the breeding of plants and animals, especially ones of agricultural values, to obtain species having a genetic makeup which results in a plant or animal having more desirable characteristics. Since the source of exogenous genetic material can be from animals or plants, synthetic equivalents of naturally occurring genetic material or totally new synthetically produced genetic material and from the same or a different species of the zygote being transformed, the invention can be used to modify a species or create a new species. Modification of a species is obtained when the genotype of the species whose zygote is being genetically transformed. A new species is obtained when the genotype of the exogenous genetic material occurs in another species and does not naturally occur in the species of the zygote being genetically transformed. For example, increased growth rate and the efficiency of feed utilization can be obtained by genetic transformation of animals used to produce meat. As an example, the genes relating to growth rate and feed utilization can be transferred from a buffalo into beef cattle which would create a new species. Dairy animals can undergo an increase in milk production and efficiency of feed utilization by transferring exogenous genetic material from species or breeds of the same species which have either or both traits. The quality and flavor of meat, for example, lamb, can also be enhanced in a similar manner. Additionally, the invention can be used as an in vivo analysis of gene expression during differentiation and in the elimination or dimunition of genetic diseases, e.g., hemophilia, Tay-Sachs disease, phenylketonuria, homocystinurea, galactosemia, thalassemia and sickle cell anemia.

Brief Description of the Drawings:

Figure 1 is a photomicrograph of microinjection of rabbit

Figure 2 is a photograph of ouchterlongy agarose gel diffusion reactions of antirabbit hemoglobin antiserum of Well A and normal nonimmunized mouse serum of Well B with a LT/Sv mouse contained in Wells 1, rabbit hemoglobin

5   preparation contained in Wells 2 and a hemoglobin preparation from mice developed from microinjected zygotes contained in Wells 3.

Figure 3 is a photograph of ouchterlongy agarose gel diffusion reactions of antirabbit hemoglobin antiserum

10   contained in Well A with dissociated rabbit hemoglobin preparation contained in Wells 1, 4 and 6, hemoglobin from a normal F, hybrid C57BL/6J X LT/Sv mouse contained in Well 5, dissociated rabbit globin chains contained in Well 2 and a hemoglobin preparation from mice developed from the β-globin

15   gene microinjected zygotes contained in Well 3.

Figure 4 is a photograph of ouchterlongy agarose gel diffusion reactions conducted in a manner identical to those of Fig. 2 except Well B contains antirabbit hemoglobin antiserum absorbed by apssage through a gel immobilized rabbit

20   hemoglobin preparation.

Embodiments of the Invention

The following definition of biological and genetic terms will be useful in understanding this invention:

"Zygote" is a diploid cell having the potential for

25   development into a complte organism. The zygote can result from parthenogensis, nuclear transplantation, the merger of two gametes by artificial or natural fertilization or any other method which creates a diploid cell having the potential for development into a complete

30   organism. The origin of the zygote can be from either the plant or animal kingdom.

"Parthenogenesis" is any technique that allows for the development of a female or male gamete into a cell and its development into an organism which technique is different from the natural development of female and male gametes.

"Genetic material" is a material containing any DNA sequence or sequences either purified or in a native state such as a fragment of a chromosome or a whole chromosome, either naturally occurring or synthetically or partially synthetically prepared DNA sequences, DNA sequences which constitute a gene or genes and gene chimeras, e.g., created by ligation of different DNA sequences. Genetic material does not include DNA sequences incorporated in or carried by a plasmid, virus or phage.

"Exogenous genetic material" is a genetic material not obtained from or does not naturally form a part of the specific germ cells or gametes which form the particular zygote which is being genetically transformed.

"DNA sequence" is a linear sequence comprised of any combination of the four DNA monomers, i.e., nucleotides of adenine, guanine, cytosine and thymine, which codes for genetic information, such as a code for an amino acid, a promoter, a control or a gene product. A specific DNA sequence is one which has a known specific function, e.g., codes for a particular polypeptide, a particular genetic trait or affects the expression of a particular phenotype.

"Gene" is the smallest, independently functional

product or controls or affects transcription and comprises at least one DNA sequence.

"Genotype" is the genetic constitution of an organism.

5   "Phenotype" is a collection of morphological, physiological and biochemical traits possessed by a cell or organism that results from the interaction of the genotype and the environment.

"Phenotypic expression" is the expression of the 10   code of a DNA sequence or sequences which results in the production of a product, e.g., a polypeptide or protein, or alters the expression of the zygote's or the organism's natural phenotype.

"Chromosome" is a fiber or threadlike structure - 15   which is completely or partically composed of genetic nucleic acid.

"Chromosomal material" is comprised of portions of or whole native chromosomes, isolated metaphase chromosomes (Wray, W., et al, Exp. Cell Res., 59:469-20   478 (1970)) or artificial chromosomes composed of native or modified histone or protamine chromosomal core complexes and DNA. Artificial chromosomes can be prepared, for example, by removing a portion of the naturally occurring DNA sequences of a native 25   chromosome and replacing it with new DNA sequences. Examples of such techniques are taught in Shewmaker, et al, Eur. J. Biochem., 107:505-510 (1980) and Cohen, et al, Eur. J. Biochem., 107:511-518 (1980).

-8-

Genetic transformation of a zygote and the organism which matures therefrom is obtained by the addition of exogenous genetic material in a manner such that the exogenous genetic material becomes a part of the nucleic portion of a zygote prior to division of the zygote. If the exogenous genetic material is added after mitosis or cellular division of the zygote, the exogenous genetic material must be added to each resulting nucleus. However, then there is the possibility that the exogenous genetic material may not be integrated into and become a part of the genetic material of the zygote and the organism which results therefrom. Thus, the exogenous genetic material can be added to any nucleic genetic material which ultimately forms a part of the nucleus of the zygote, including the zygote nucleus. The nucleic genetic material must be in a physical state which enables it to takeup the exogenous genetic material. There are numerous ways for accomplishing this. For example, the exogenous genetic material can be placed in the nucleus of a ‑primordial germ cell which is deploid, e.g., a spermatogonium or oogonium. The primordial germ cell is then allowed to mature to a gamete which is then united with another gamete or source of a haploid set of chromosomes to form a zygote.

The exogenous genetic material can be placed in the nucleus of one of the gametes, e.g., a mature sperm, egg or polar body, which forms a part of the zygote. In this instance, if the exogenous genetic material is placed in the mature sperm prior to fertilization, the sperm cell should be induced to undergo decondensation. Otherwise, the chromosomal complement of the sperm cell is too dense to allow the addition of any material to its nucleus. Techniques for the decondensation are known in the art. Examples of such techniques can be found in Mahi, D.A., et al, J. Reprod. Fert., 44:293-296 (1975); Hendricks, D.M. et al, Exptl. Cell Res., 40:402-412 (1965); and Wagner, T.E., et al, Archives

use of a disulfide reductant is preferred. The sperm cell into
which the exogenous genetic material is placed is thereafter
placed in the egg to enable the formation of the zygote.

5   The exogenos genetic material can be placed in the
nucleus of the mature egg. It is preferred that the egg be in
a fertilized or activated, by parthenogenesis, state. After
the addition of the exogenous genetic material, then a
complementary haploid set of chromosomes, e.g., a sperm cell
or polar body, is added to enable the formation of zygote.
10 When a polar body is used to diploidize a haploid egg, the
exogenous genetic material can be placed in the polar body.

It is preferred that the exogenous genetic material be
placed in either the male or female pronucleus of the zygote.
More preferably, it is placed in either the male or the female
15 pronucleus as soon as possible after the sperm enters the
egg. In other words, right afte the formation of the male
pronucleus when the pronuclei are clearly defined and are
well separated, each being located near the zygote membrane.
The male pronucleus is the preferred site for addition of the
20 exogenous genetic material. When the exogenous genetic
material is inserted into the female pronucleus, it is preferred
that the exogenous genetic material be chromosomal material,
more preferably that the chromosomal material be comprised of
histone chromosomal core complexes and DNA, which can be
25 artificial chromosomal material reconstituted from DNA
sequences of native or modified histone chromosomal core
complexes.

It is most preferred that the exogenous genetic material
be added to the male DNA complement, or a DNA complement
30 other than the DNA complement of the female pronucleus, of
the zygote prior to its being processed by the ovum nucleus
or the zygote female pronucleus. It is thought that the ovum
nucleus or female pronucleus release molecules which affect

-10-

the male DNA complement, perhaps by replacing the protamines of the male DNA with histones, thereby facilitating the combination of the female and male DNA complements to form the diploid zygote. Thus, for the purpose of this

5 invention, the processing of a DNA complement by the female pronucleus of the zygote refers to a biochemical effect the female pronucleus has on the DNA complement prior to the physical contact between the pronuclei or between the female pronucleus and the particular DNA complement. Thus, it is

10 preferred that the exogenous genetic material be added to the male complement of DNA or any other complement of DNA prior to its being affected by the female pronucleus. For example, the exogenous genetic material is added to the early male pronucleus, as soon as possible after the formation of

15 the male pronucleus, which is when the male and female pronuclei are well separated and both are located close to the cell membrane. Alternatively, the exogenous genetic material could be added to the nucleus of the sperm after it has been induced to undergo decondensation. Sperm containing the

20 exogenous genetic material could then be added to the ovum or the decondensed sperm could be added to the ovum with the exogenous genetic material being added as soon as possible thereafter. Even when the DNA complement to the ovum DNA is of a female source, it is preferred that the

25 exogenous material be added to that female DNA complement shortly after its placement within the ovum or prior to its placement in the ovum in order to allow that DNA complement to be processed by the female pronucleus.

For the purpose of this invention a zygote is essentially

30 the formation of a diploid cell which is capable of developing into a complete organism. Generally, the zygote will be comprised of an egg containing a nucleus formed, either

-11-

naturally or artifically, by the fusion of two haploid nuclei
from a gamete or gametes. Thus, the gamete nuclei must be
ones which are naturally compatible, i.e., ones which result
in a viable zygote capable of undergoing differentiation and
developing into a functioning organism. Generally, a euploid
zygote is preferred. If an aneuploid zygote is obtained, then
the number of chromosomes should not vary by more than one
with respect to the euploid number of the organism from
which either gamete originated.

In addition to similar biological considerations, physical
ones also govern the amount of exogenous genetic material
which can be added to the nucleus of the zygote or to the
genetic material which forms a part of the zygote nucleus. If
no genetic material is removed, then the amount of exogenous
genetic material which can be added is limited by the amount
which will be absorped without being physically disruptive.
Generally, the volume of exogenous genetic material inserted
will not exceed about 10 picoliters. Obviously, more
exogenous genetic material can be added if it is exchanged
for existing genes. Then, to a certain extent, the amount
added is unlimited as long as the number of existing genes
being removed is equal to the number inserted. However,
the physical effects of deletion and addition must not be so
great as to physically destroy the viability of the zygote.
The biological limit of the number and variety of DNA
sequences will vary depending upon the particular zygote and
functions of the exogenous genetic material and will be
readily apparent to one in the art, because the genetic
material, including the exogenous genetic material, of the
resulting zygote must be biologically capable of initiating and
maintaining the differentiation and development of the zygote
into a functional organism. From a biological consideration,

relating to mitosis, when the exogenous genetic material is a chromosome, the amount inserted will not be greater than one chromosome and preferably will be less than one chromosome.

The number of copies of the DNA sequences which are added to the zygote is dependent upon the total amount of exogenous genetic material added and will be the amount which enables the genetic transformation to occur. Theoretically only one copy is required; however, generally, numerous copies are utilized, for example, 1,000-20,000 copies of a gene, in order to insure that one copy is functional. Generally, there is no advantage to having more than one functioning copy of each of the inserted exogenous DNA sequences unless an enhanced phenotypic expression of the exogenous DNA sequences is desired. There are instances where more than one functional copy of the exogenous DNA sequences may not be desirable, for example, when the exogenous DNA sequences work in conjunction with endogenous DNA sequences of the organism to produce a particular product. A specific example, in the case of a mammal, is the insertion of sufficient copies of an exogenous gene coding for β-globin (without concommitant insertion of the α-globin gene) to produce many functional β-globin genes in the genetically transformed organism. This can cause the organism to have a form of thalassemia due to a disproportionate production of β-globin compared to α-globin.

The particular composition or form of the exogenous genetic material is not critical. If a particular trait is desired to be incorporated into an organism, then the genetic material needs to contain the DNA sequence or sequences, or gene or loci, which code for the trait. Thus, whether the exogenous genetic material is a whole chromosome, a portion of a chromosome, unpurified native DNA or synthetic or semi-synthetic DNA or chromosomal complexes is dependent upon the particular trait or traits sought to be incorporated into

obtaining DNA sequences by gene excising, splicing, synthesis, isolation, purification and cloning as well as enzymes used in such processes, vehicles and hosts for cloning of recombinant DNA, screening and selection of
5   cloned DNA and detection and analysis of expression of cloned genes are known in the art. Methods in Enzymology: Recombinant DNA, Vol. 68, edited by Ray Wu, Academic Press, Inc., New York, 1979, is an example of a reference which discusses many of these techniques.
10   Depending upon the particular trait or traits being inserted into the zygote, it maybe desirable to include the control sequences of the genetic material responsible for controlling the gene(s) coding for the trait, particularly when the genotype of the zygote contains no similar gene
15   whose control region could be used to regulate the gene or when greater genetic activity is desired. In some instances it may be desirable, to include a control region, e.g., an artifical one spliced to the exogenous gene, which will activate the gene when the organism is exposed to a different
20   stimulus other than the natural stimulus of the gene. For example, a growth hormone gene inserted into a bovine zygote could be attached to a control gene which is responsive to a molecule which could be added to the feed of the animal. Thus, each time the animal ingested the
25   molecule, the growth hormone gene would be activated.
          Techniques for obtaining immature germ cells, gametes and zygotes, for parthogenesis and for in vitro and in vivo development of zygotes are well known. The conditions under which the exogenous genetic material is added or
30   inserted should be such as to maintain the cells in a viable state and will grossly mimic the natural in vivo development conditions of the cells. After the exogenous genetic material is inserted and the zygote formed, the zygote is allowed to develop under in vitro conditions. In the case of some animal

zygotes, for example, mammals, it will be necessary to complete the development of the zygote in vivo by implanting it into a foster mother. It is preferred that the implantation occurs at the morula or blastocyst stage of development.

5   Any technique which allows for the addition of the exogenous genetic material into nucleic genetic material can be utilized so long as it is not destructive to the cell, nuclear membrane or other existing cellular or genetic structures. The exogenous genetic material is preferentially inserted into

10   the nucleic genetic material by microinjection. Microinjection of cells and cellular structures is known and is used in the art.

EXAMPLE

Procedure:

15   Fertilized eggs at early pronuclear stage, the male and female pronuclei being separated and distinguishable within the cytoplasm, as shown in Figure 1, were collected from the oviducts of C57BL/6J female mice which had been mated to LT/Sv males. After removing the surrounding cumulus cells

20   in culture medium containing I milligram (mg) bovine testis hyaluronidase per milliliter (ml) culture medium, as described in Biol. Reprod., 8:420-426 (1973), pooled zygotes from several females were wased in fresh medium and stored, until micromanipulation, in a drepression slide containing culture

25   medium overlayered with paraffin oil in an atmosphere of 5 percent carbon dioxide, 5 percent oxygen and 90 percent nitrogen (percentages are based on volume) at 37° C.

          The hybrid plasmid Z-pCR1/Rchr βG-1, containing rabbit β-globin, was extracted from E. Coli HB101 containing

30   the hybrid plasimd using a lysozyme, Triton x-100 ethylene-diaminetetracetic acid lysis procedure and was subsequently purified by cesium chloride-Ethidium bromide density

-15-

gradient cetrifugation followed by agarose gel electrophoresis in a low melting point agarose. The purified plasmid which contained the rabbit β-globin gene was then digested with Hha I restriction endonuclease (Plasmid pCR1 has numerous

5   Hha I sites while the β-globin has none). The 6200 base pair β-globin gene DNA fragment was then purified by electrophoresis of the Hha I digest of the plasmid in low melting point agarose. The β-globin gene fragment DNA band was extracted fromthe agarose by melting the gel at 60° C

10   followed by extraction with phenol. The thus obtained β-globin gene fragments contained approximately 50 micrograms of DNA per milliliter. The β-globin gene sequence contained a 223 base pair control sequence upstream from the cap site of the structural gene which included a TATA sequence

15   (Goldberg, M., Ph.D. thesis, Stanford University (1979) and the CAAT sequences (Efstratiadis, A., et al, Cell, 21:653-668 (1980).

       Injection pipets with an external diameter of about 1 micrometer and holding pipets with an external diameter of

20   about 60-70 micrometers were prepared from pyrex tubing as described in Proc. Natl. Sci. U.S.A., 74:5657-5661 (1977). Manipulation of the pipets for holding and subsequence injection of the zygote was accomplished using Leitz micro-manipulators and paraffin oil filled Hamilton microsyringes. A

25   small drop each of culture medium with 5-6 zygotes and of rabbit β-globin chromosomal gene solution were placed on a special microscope slide and covered with paraffin oil. Approximately 10 picoliters of β-globin gene solution, which is approximately 20,000 DNA sequences, was drawn into the

30   injection pipet which was then moved to the drop of culture medium containing the eggs. A zygote was positioned onto the holding pipet so that the male pronucleus was in juxtaposition to the injection pipet for subsequent injection of the β-globin gene suspension into the pronucleus. After

-16-

the drop of medium on the microscope slide and placed in culture tubes where they were allowed to develop for five days. The conditions of the preimplantation development is described in <u>Biol. Reprod.</u>, 8:420-426 (1973). Eggs
5   developing in morulae or blastocysts were transplanted into uteri of F_1 hybrid, C57BL/6J X SJL/J, foster mouse mothers who were at day three of pseudopregnancy. The foster mothers carried the implanted embryos to term. The preimplantation and postimplantation development of F_1
10 hybrid, C57BL/6J X LT/Sv zygotes after microinjection of rabbit β-globin chromosomal gene into the male pronucleus is given in Table 1.

TABLE 1

15 Number zygotes injected	143
Number eggs cleaving	139
Number morulae and blastocysts transplanted to foster mothers	120
Number foster mothers	12
20 Number foster mothers pregnant	8
Number offspring born	28[a]

[a]Eighteen males and ten females

Analyses:
25   The presence of rabbit β-globin DNA sequences in the nucleic genetic material of mice developed from the rabbit β-globin gene microinjected F_1 hybrid mouse zygote was demonstrated by the specific hybridization of plasmid Z-pCR1/RchrβG-1 to the liver DNA of these mice.

-17-

Hemoglobin was prepared from blood samples taken from each of the mice developed from the rabbit β-globin gene microinjected F_1 hybrid mouse zygotes and analyzed by agarose immunodiffusion against F_1 hybrid (C57BL/6JxLT/Sv)

5   mouse antirabbit hemoglobin antiserum. The three samples which showed the most distinct positive reaction with the antiserum were pooled and used in the immunodiffusion analyses hereinafter described.

Agarose gel immunodiffusion, i.e., Figure 2, using F_1·

10   hybrid mouse antirabbit hemoglobin antiserum showed a major band of precipitation with the control rabbit hemoglobin, a major band of precipitation with the hemoglobin prepared from the F_1 hybrid mice that developed from zygotes microinjected with purified rabbit β-globin gene sequences and no evidence

15   of precipitation with hemoglobin prepared from normal F_1 hybrid mice. The major precipitation band between the microinjected mouse hemoglobin and the mouse antirabbit hemoglobin antiserum established that the erythrocytes of the mice developed from the β-globin gene microinjected zygotes

20   contain a molecular species which reacts specifically with antiserum produced against rabbit hemoglobin.

The agarose gel immunodiffusion of Figure 3 shows identity between one of the dissociated rabbit globin chains and a molecular species present in the erythrocytes of mice

25   developed from the β-globin gene microinjected zygotes.

In order to establish that the immunoprecipitation band between the F_1 hybrid mouse antirabbit hemoglobin antiserum and the hemoglobin preparation from mice resulting from zygotes microinjected with rabbit β-globin genes is the result

30   of a specific immunological reaction, a control immunodiffusion analysis was performed using F_1 hybrid mouse antirabbit hemoglobin antiserum which had been absorbed by passage

-18-

through a column containing rabbit hemoglobin bound to an inert matrix. The absorbed antiserum, as shown in Figure 4, did not react with either rabbit hemoglobin or the hemoglobin from the mice which developed from themicroinjected zygotes.

5   In addition to the immuno analyses described above, the hemoglobin of the mice that had been genetically transformed with rabbit β-globin gene was isoelectrically focused. The isoelectric focusing revealed that their hemoglobin contained a subtraction which is not the same as the hemoglobin of the

10   control mice or of a rabbit and thus appears to be a new hemoglobin. In view of the isoelectric focusing results and the immunological activity of the hemoglobin of the genetically transformed mice with the mouse antirabbit hemoglobin antiserum, the hemoglobin of the benefically transformed mice

15   is comprised apparently of at least one rabbit β-globin and mouse α-globins.

The mice that had been genetically transformed with the rabbit β-globin gene also have an abnormally high percent, about 6 percent, of reticulocytes in their blood. The control

20   mice of the same inbred species and age have a reticulocyte concentration of about 0.5 percent of their blood cells. The high percentage of reticulocytes is indicative of thalassemia, an anemia resulting from the presence of excess rabbit β-globin.

25   Finally, several of the mice that were genetically transformed by the rabbit β-globin gene have produced offspring which also phenotypically express the rabbit β-globin gene.

Preparation of Hemoglobins and Antisera used in the
30   Immunological Analyses:

Each hemoglobin preparation was prepared as a purified hemolysate with blood from New Zealand white rabbits, normal F_1 hybrid (C57BL/6JxLT/Sv) mice and F_1 hybrid

(C57BL/6JxLT/Sv) mice developed from zygotes microinjected with rabbit β-globin gene DNA sequences. The erythrocytes for each hemoglobin preparation were washed exhaustively with phosphate buffered saline, lysed in distilled water and centrifuged at 12,000 times gravity for 10 minutes to remove the erythrocyte membrane. Prior to application of the hemoglobin sample to agarose immunodiffusion plates, the hemoglobin solutions were filtered through a 0.45 micro filter and adjusted toa concentration of 100 mg/ml.

The mouse antirabbit hemoglobin antiserum was raised in F_1 hybrid (C57BL/6JxLT/Sv) mice. Rabbit hemoglobin in distilled water was emulsified with an equal volume of complete Freund's adjuvant and each animal received a 500 microgram priming dose, subcutaneously, divided among four sites. After one and two weeks all animals received second and third injections of 10 micrograms of rabbit hemoglobin emulsified in incomplete Freund's adjuvant administered subcutaneously and intraperitoneally, respectively. After four weeks each animal was injected intraperitoneally with 600 micrograms of rabbit hemoglobin in complete Freund's adjuvant. The immunized animals were bled after week five and the serum separated, tested against rabbit hemoglobin and preserved in the presence of sodium azide.

Free rabbit globin chains were prepared from a rabbit hemolysate by acetone extraction of the heme iron complex resulting in dissociation of the α and β protein subunits. Five hundred micrograms of packed, washed, rabbit erythrocytes were lysed in 2 milliliters of distilled water and the resulting hemolysate dripped slowly into a stirred solution of acetone and 1.2 milliliters of concentrated HC1 at -70° C. The resulting precipitated rabbit globin chains were allowed to stand for 15 minutes at -70° C in the extraction mixture

after which they were washed twice with -20° C acetone and dried under vacuum at liquid nitrogen temperatures. The dried precipitate was dissolved in 2 milliliters of distilled H_2O and dialysed exhaustively against phosphate buffered saline.

5   Rabbit hemoglobin, 30 milligrams, was coupled to Sepharose 4B Gel beads by reaction with 1 gram washed CNBr-activated Sepharose 4B from Pharmacia Fine Chemicals, Inc. in 0.1M $NaHCO_3$ and 0.5M NaCl at a pH of 8.0, for 3 hours at room temperature. Following the coupling reaction,

10   the remaining active groups on the gel beads were reacted with 1M Tris, pH 8.0, and the hemoglobin Sepharose gel was washed exhaustively to remove any unbound hemoglobin. The gel-immobilized rabbit hemoglobin was used to prepare a miniature column for the selective removal of antirabbit

15   hemoglobin antibodies from immunized mouse serum.

Reactivity of transformed mouse hemoglobin antigens with mouse antirabbit hemoglobin antiserum was detected by double diffusion analysis on 1 percent agarose slides. Antigen wells contained 15 microliters of each hemoglovin

20   antigen (100 mg/ml) and the antibody well contained 15 microliters of antirabbit hemoglobin antiserum. Each well was refilled twice during the 24 hour incubation at 37° C. Reactivity was characterized by the production of a band of precipitation between the hemoglobin of the transformed mice

25   and the mouse antirabbit hemoglobin antiserum.

This invention has been described with particular reference to a specific embodiment thereof, but it will be understood that variations and modifications can be effected within the spirit of the invention.

CLAIMS

What is Claimed is:

1. A method for obtaining genetic transformation of an organism, including its zygote, comprising placing exogenous genetic material into the nucleus of the organism's zygote or into genetic material which forms a part of the nucleus of the zygote and allowing the zygote to develop into the organism.

2. A method for obtaining genetic transformation of an animal, including its zygote, comprising placing exogenous genetic material into the nucleus of the animal's zygote or into genetic material which forms a part of the nucleus of the animal's zygote and allowing the zygote to develop into the organism.

3. The method of Claim 1 or Claim 2 wherein the exogenous genetic material is placed into a nucleus of a gamete which forms a part of the zygote.

4. The method of Claim 3 wherein the gamete is a sperm.

5. The method of Claim 3 wherein the gamete is an egg.

6. The method of Claim 1 or Claim 2 wherein the exogenous genetic material is placed in a pronucleus of the zygote.

7. The method of Claim 1 or Claim 2 wherein the exogenous genetic material is placed in the nucleus of the sperm before male pronuclear formation.

8. The method of Claim 6 wherein the exogenous genetic material is placed in the male pronucleus prior to complete pronuclear formation.

9. The method of Claim 6 wherein the exogeneous genetic material is placed in the female pronucleus.

10. The method of Claim 9 wherein the exogenous genetic material placed in the female pronucleus is comprised

11.   The method of Claim 10 wherein the chromosomal material is comprised of DNA associated with a histone chromosomal core complex.

12.   The method of Claim 1 or Claim 2 wherein at least a portion of the exogenous genetic material codes for a protein.

13.   The method of Claim 12 wherein the zygote or the organism which develops from the zygote phenotypically expresses the exogenous DNA sequences.

14.   A method for obtaining genetic transformation of an organism, including its zygote, wherein a female pronucleus forms at least a portion of the nucleus of the zygote, comprising placing exogenous genetic material into genetic

5   material which forms a part of the nucleus of a zygote prior to the genetic material coming into physical contact with the female pronucleus of the zygote.

15.   A method for obtaining genetic transformation of an animal, including its zygote, wherein the female pronucleus forms at least a portion of the nucleus of the zygote, comprising adding exogenous genetic material to genetic

5   material which forms a part of the nucleus of a zygote prior to the genetic material coming into physical contact with a female pronucleus of the zygote.

16.   The method of Claim 14 or Claim 15 wherein the exogenous genetic material is added to the nucleus of a sperm which sperm ultimately contributes to the formation of the zygote.

17.   The method of Claim 14 or Claim 15 wherein the exogenous genetic material is added to the zygote's male pronucleus.

18.   The method of Claim 17 wherein the exogenous genetic material is added to the male pronucleus shortly after the formation of the male pronucleus when it is clearly defined and separated from the female pronucleus of the

5   zygote.

19. The method of Claim 15 wherein the animal is a mammal and the exogenous genetic material is added to the zygote's male pronucleus.

20. The method of Claim 15 wherein the animal is a mammal and the exogenous genetic material is added to the male pronucleus shortly after the formation of the male pronucleus when it is clearly defined and separated from the female pronucleus of the zygote.

21. A process for obtaining a new species comprising inserting exogenous genetic material into genetic material which forms at least a part of a nucleus of a zygote, wherein the exogenous genetic material contains at least one genotype not possessed by the genotype of the zygote or members of the zygote's species thereafter allowing the zygote to undergo differentiation and development into an organism of the new species.

22. A method for obtaining a modified species comprising inserting exogenous genetic material into genetic material which forms at least a part of a nucleus of a zygote of the species being modified, wherein the genotype of the exogenous genetic material is found naturally in the genotype of at least one member of the zygote's species and thereafter allowing the zygote to undergo differentiation and development into a mature organism.

23. A method for genetically transforming a mammal comprising inserting exogenous genetic material into the nucleus of the mammal's zygote or into any genetic material which forms at least a part of the nucleus of the mammal's zygote and thereafter allowing the zygote to undergo differentiaton and development into the genetically transformed mammal.

24. The method of Claim 23 wherein the genetic transformation results in a phenotypic expression of at least a portion of the exogenous genetic material.

25.   The method of Claim 23 wherein the exogenous genetic material is inserted into a pronuclei of the zygote prior to their fusion.

26.   The method of Claim 19 wherein the exogenous genetic material is inserted into the male pronucleus prior to the male pronucleus being processed by the female pronucleus.

27.   A method for genetically transforming an organism comprising inserting exogenous genetic material containing specific DNA sequences which code for a desired transformation of the organism into the nucleus of the organism's

5   zygote or any genetic material which forms at least a part of the nucleus of the organism's zygote and thereafter allowing the zygote to undergo differentiation and development into a mature organism.

28.   The method of Claim 27 wherein the DNA sequences code for the production of a growth hormone.

29.   A genetically transformed organism obtained by placing exogenous genetic material into the nucleus of a zygote or into genetic material which forms a part of the nucleus of the zygote and allowing the zygote to develop into

5   the genetically transformed organism.

30.   A new species obtained by placing exogenous genetic material into genetic material which forms at least a part of a nucleus of a zygote, wherein the exogenous genetic material contains at least one genotype not possessed by the

5   genotype of the zygote's species, thereafter allowing the zygote to undergo differentiation and development into an organism of the new species.

31.   A modified species obtained by placing exogenous genetic material into nucleic genetic material which forms the nucleus of a zygote, wherein the genotype of the exogenous genetic material is found in the genotype of at least one

5   member of the zygote's species, and thereafter allowing the

32. The species of Claim 30 or Claim 31 obtained by placing the exogenous genetic material into the genetic material which forms a part of the nucleus of the zygote prior to the genetic material being processed by a female
5   pronucleus.

33. A mouse containing rabbit β-globin gene.

34. A mouse containing rabbit β-globin gene wherein the mouse phenotypically expresses the rabbit β-globin and is capable of transmitting the rabbit β-globin genotype and phenotype to its offspring.

35. The mouse of Claim 34 which produces the rabbit β-globin in its erythrocytes.

36. A process for producing a mouse which genotypically and phenotypically expresses a rabbit β-globin gene comprising inserting into a pronucleus of the mouse's zygote exogenous genetic material which contains the gene for
5   rabbit β-globin, thereafter allowing the pronuclei to fuse to form the nucleus of the zygote and allowing the zygote to undergo differentiation and development into a mouse.

37. The process of Claim 29 wherein the exogenous genetic material containing the gene for rabbit β-globin is inserted into the male pronucleus shortly after the formation of the male pronucleus when it is clearly defined and sep-
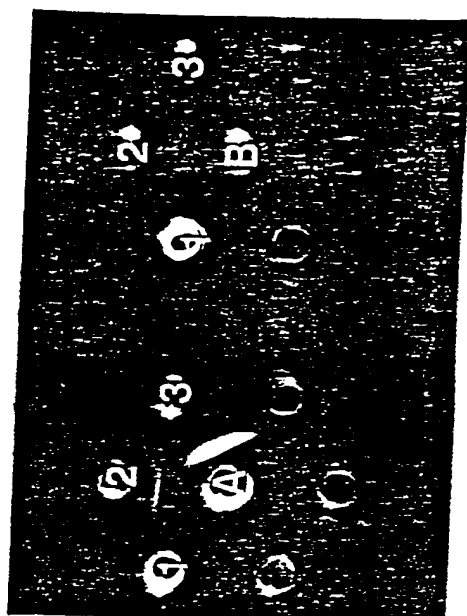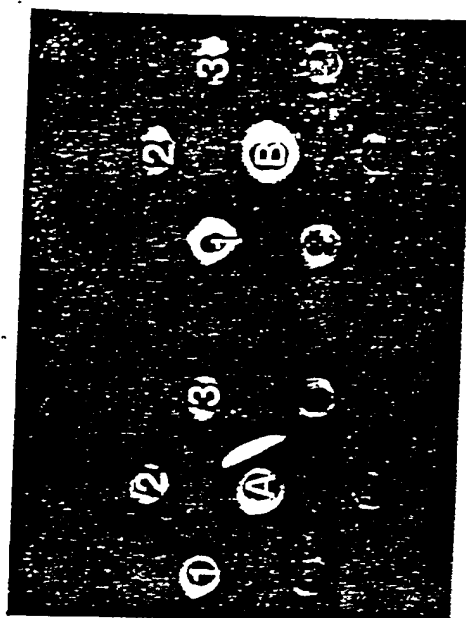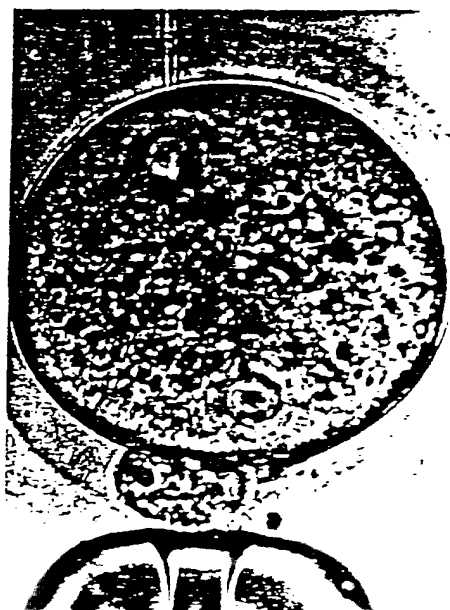5   arated from the female pronucleus of the zygote.
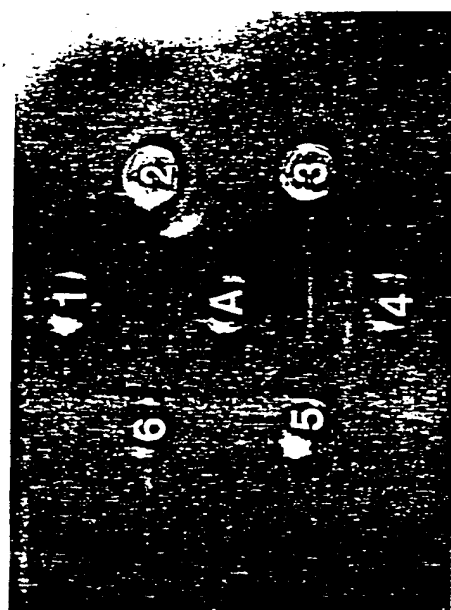
FIG.2

FIG.4

FIG.3

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL³ C12P 21/00, 21/02; C12N 15/00

U.S. CL. 435/68,70,172

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴

Classification System	Classification Symbols
U.S.	435/68,70,172

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁵

COMPUTER — BIOSIS FILE: AND FILE 55

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X	N, PROC. NATL. ACAD. SCI. U.S.A. 77:7380-7384(1980).	1-32
Y	N, NATURE VOLUME 281, PP. 544-548, 18 OCTOBER 1979.	28
Y	N, NATURE VOLUME 277, PP. 108-114, 11 JANUARY 1979.	1-27, 29-37
X	N, NATURE VOLUME 284, PP. 422-425, 03 APRIL 1980.	33

* Special categories of cited documents: ¹⁵

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ²

Date of Mailing of this International Search Report ²